The American Journal of Pathology, Vol. 175, No. 2, August 2009

DOI: 10.2353/ajpath.2009.080920

Tumorigenesis and Neoplastic Progression

Expression of Integrin $\alpha6\beta1$ Enhances Tumorigenesis in Glioma Cells

Estelle Delamarre, Salma Taboubi, Sylvie Mathieu, Caroline Bérenguer, Véronique Rigot, Jean-Claude Lissitzky, Dominique Figarella-Branger, L'Houcine Ouafik, and José Luis

From INSERM UMR 911, and Aix-Marseille Université, Marseille, France

The integrin $\alpha6\beta1$ and its main ligand laminin-111 are overexpressed in glioblastoma, as compared with normal brain tissue, suggesting they may be involved in glioblastoma malignancy. To address this question, we stably expressed the $\alpha6$ integrin subunit in the U87 cell line via retroviral-mediated gene transfer. We show that cell surface expression of the $\alpha6\beta1$ integrin led to dramatic changes in tumor U87 cell behavior, both *in vitro* and *in vivo*. Nude mice receiving either subcutaneous or intracerebral inoculation of $\alpha6\beta1$-expressing cells developed substantially more voluminous tumors than mice injected with control cells. The difference in tumor growth was associated with a marked increase in vascularization in response to $\alpha6\beta1$ integrin expression and may also be related to changes in the balance between cell proliferation and survival. Indeed, expression of $\alpha6\beta1$ enhanced proliferation and decreased apoptosis of U87 cells both in the tumor and *in vitro*. Additionally, we demonstrate that $\alpha6\beta1$ is implicated in glioblastoma cell migration and invasion and that laminin-111 might mediate dissemination of $\alpha6\beta1$-positive cells *in vivo*. Our results highlight for the first time the considerable role of the integrin $\alpha6\beta1$ in glioma progression. *(Am J Pathol 2009, 175:844–855; DOI: 10.2353/ajpath.2009.080920)*

Malignant brain tumors have an increasing incidence in both children and adults. In adults, the most common type of primary brain tumor, malignant glioma, is considered as one of the deadliest of human cancers. Despite recent advances in both diagnostic modalities and therapeutic strategies, the 5-year survival rate of less than 3% in patients with glioblastoma is among the lowest for all cancers.[1] Patients with the most malignant histopathological subtype, glioblastoma, carry the worst prognosis, with median survival rate of less than 1 year, despite aggressive surgery associated with adjuvant radiotherapy and chemotherapy.[1] Glioblastoma are characterized by rapidly dividing cells, high degree of vascularity, invasion into normal brain tissue, and an intense resistance to death-inducing stimuli.[2,3] Since integrins, the major family of extracellular matrix (ECM) receptors, are involved in these events, they are one of the most promising molecules to consider for a targeted therapy.

Integrins are cell surface transmembrane $\alpha\beta$ heterodimers that recognize specific ECM ligands. The combination of α and β subunits, leading to the formation of at least 24 receptors, determines the ligand specificity.[4] Glioblastoma commonly displays enhanced expression of several integrins along with their ECM ligands: $\alpha v\beta3$ and $\alpha v\beta5$ (tenascin and vitronectin receptors), $\alpha5\beta1$ (fibronectin receptor), $\alpha2\beta1$ (collagens receptor), and $\alpha3\beta1$, $\alpha6\beta4$, and $\alpha6\beta1$ (laminins receptors).[5] Numerous studies have focused on the αv integrin family. The integrins $\alpha v\beta3$ and $\alpha v\beta5$ are markers of glioblastoma malignancy[6] and influence a variety of processes in glioblastoma progression *in vivo*, including proliferation, apoptosis, and angiogenesis.[7] Furthermore, cilengitide, an $\alpha v\beta3$ and $\alpha v\beta5$ integrins antagonist, extends mouse survival by delaying the tumor growth[8,9] and is nowadays in clinical trial for recurrent malignant glioma. Two other integrins, $\alpha5\beta1$ and $\alpha3\beta1$, have been shown to be implicated in glioma cell adhesion and migration *in vitro.*[10,11] In addition, the use of $\alpha5\beta1$ antagonists reduces glioma cell proliferation *in vitro,*[10] while $\alpha3\beta1$ antagonists inhibited glioma invasion *in vivo.*[11]

The $\alpha6$ integrin subunit associates with $\beta1$ or $\beta4$ subunits to form functional heterodimers that selectively bind laminins. The $\alpha6\beta4$ integrin is essential for the organiza-

Supported in part by grants from the Association pour la Recherche contre le Cancer (ARC) and the "Cancéropôle Provence-Alpes-Côte d'Azur" (Réseau Structurant RS019).

Accepted for publication May 7, 2009.

Supplemental material for this article can be found on *http://ajp.amjpathol.org.*

Address reprint requests to José Luis, INSERM U911 (CRO2), Faculté de Pharmacie, 27, Bd J. Moulin, 13 385 Marseille Cedex 5, France. E-mail: jose.luis@pharmacie.univ-mrs.fr.

tion and maintenance of epithelial hemidesmosomes that link the intermediate filaments with the extracellular matrix.[12] The major ligand of α6β4 is the laminin-332, while α6β1 is a well-characterized laminin-111 receptor. Overexpression of α6β1 integrin has been associated with the progression of many epithelial tumors. In particular, induction of α6β1 expression is an early event in hepatocellular carcinogenesis.[13,14] In the same way, during prostate cancer progression α6β1 is continually expressed and found in micrometastases.[15] Expression of α6β1 integrin has also been linked to metastatic potential of melanoma cells,[16] and has been involved in the survival and metastatic potential of human breast carcinoma cells.[17,18] Moreover, in a recent study using the α6-blocking antibody GoH3, Lee et al[19] inhibited angiogenesis and breast carcinoma growth *in vivo*.

Several studies concerning gliomas and the α6β1 ligand laminin-111 have been reported in the literature. Using immunohistochemistry studies, Gingras et al[20] showed that α6 integrin was strongly expressed in glioblastoma tissue, whereas it was weakly expressed in normal brain. Previtali et al[21] confirmed that the expression of α6 was increased in glioblastoma and in other central nervous system tumors, such as meningioma, astrocytoma, and neuroblastoma, when compared with the autologous normal tissue counterpart. In glioblastoma biopsies, laminin-111 is highly expressed on tumor blood vessels, but also within the brain tumor as punctuate deposits and at the tumor invasion front.[22] *In vitro*, glioma cells can both secrete laminin-111 and induce its expression in normal brain tissue.[22–24] Moreover, laminin-111 is one of the most permissive substrates for adhesion and migration of glioma cells *in vitro.*[25–27] Additionally, over laminin-111, migrating glioma cells are protected from apoptosis.[28] For all these reasons, we hypothesized that laminin-111 and its main receptor α6β1 may contribute to glioblastoma progression.

In the present study we investigated the role of integrin α6β1 in glioblastoma malignancy by using U87, a well-characterized glioblastoma cell line. We report that stable expression of α6β1 in this α6-negative cell line leads to enhanced tumor progression and tumor growth *in vivo*. We demonstrate that α6β1 is pro-angiogenic and acts on the balance between proliferation and apoptosis. Additionally, we show that α6β1 is involved in glioblastoma cell migration and invasion. Our results highlight for the first time the considerable role of integrin α6β1 in the malignant phenotype of glioblastoma cells and demonstrate that the α6β1-expressing cell is an appropriate model for the study of glioblastoma progression.

Materials and Methods

Reagents

Eagle's minimal essential medium was purchased from Lonza (Levallois-Perret, France) and fetal calf serum from Invitrogen (Cergy Pontoise, France). Mouse laminin, 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were from Sigma (St Quentin Fallavier, France). Matrigel and rat tail type I collagen were from BD Biosciences (Le Pont de Claix, France). Mouse monoclonal antibodies (mAb) Lia1/2 and K20 (anti-β1) and rat mAb GoH3 (anti-α6) were from Beckman Coulter (Marseille, France). Mouse mAb 3E1 and rabbit polyclonal antibody AB1922 (anti-β4) were from Millipore (St Quentin en Yvelines, France). Rabbit polyclonal antibodies anti-β1 and anti-α6 were produced in our laboratory. Rabbit antibody anti-mouse laminin α1 chain (1057+) was a gift from Dr. Takako Sasaki.[29] Antibodies to factor VIII-related antigen and Ki-67 nuclear antigen were purchased from DAKO (Glostrup, Denmark). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and enhanced chemiluminescence reagents were from GE Health care (Orsay, France). Secondary antibodies conjugated with AlexaFluor were from Invitrogen.

Cell Culture and Retroviral Transduction

U87 cells (ATCC) and the other human glioblastoma cell lines were routinely cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 2 mmol/L glutamine. U87 variants were obtained by retroviral strategy. Retroviral vectors were obtained by ligation of human α6 cDNA (a kind gift of Dr. A. Sonnenberg, Cancer Institute, Amsterdam, The Netherlands) in the LZRS-IRES-EGFP retroviral vector by appropriate molecular biology procedures. Defective retroviral particles for infection of the U87 cell line were obtained from conditioned media of the Phoenix amphotropic retrovirus-producing cell line after transfection with LZRS-IRES-EGFP or LZRS-α6-IRES-EGFP plasmids. The mock-U87 (control) and α6-U87 (α6-expressing) cell lines were obtained after serial infections of U87 cells with particles shuttling the empty or the α6-containing vector, respectively, followed by fluorescence-activated cell sorting (FACS) for high enhanced green fluorescent protein expression and α6 expression. Cell surface expression of integrin subunits was measured using a Becton-Dickinson FACScan flow cytometer as already described.[30]

Co-Immunoprecipitation

Cells were washed twice with ice-cold PBS and solubilization was performed by a 30-minute incubation at 4°C with 50 mmol/L Tris-HCl pH8, 200 mmol/L NaCl, and 1% Triton X-100 (radioimmunoprecipitation assay buffer) containing 0.5% bovine serum albumen and a mixture of proteinase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 500 U/ml aprotinin, 1 μg/ml leupeptin, 1 μmol/L pepstatin, 1 mmol/L iodoacetamide, and 1 mmol/L ortho-phenantrolin). Equal amounts of clarified cell lysates were incubated with 5 μg of anti-α6 mAb (GoH3) or anti-β1 mAb (K20) overnight at 4°C. After adding protein G-agarose beads for 1 hour, the suspension was centrifuged and pellets were washed three times with radioimmunoprecipitation assay buffer, 2 times in radioimmunoprecipitation assay buffer containing 500 mmol/L NaCl and once with PBS. Immunoprecipitated proteins

were solubilized in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted with polyclonal antibodies to α6, β1, or β4 integrin subunits. Bound antibodies were revealed by horseradish peroxidase-conjugated anti-rabbit antibodies and the ECL detection system.

Cell Adhesion and Spreading Assays

Adhesion and spreading assays were performed as previously described.[30] Briefly, cells in single-cell suspension were added to wells coated with 10 μg/ml of purified ECM proteins and allowed to adhere (30 minutes) or spread (60 minutes) to the substrata at 37°C. After washing, attached cells were fixed, stained by 0.1% crystal violet. For adhesion assays crystal violet was then solubilized in 1% SDS and quantified by absorbance at 600 nm. For spreading assays, 10 random fields per well were pictured and the percentage of spread cells was quantified by counting the number of round cells. The cell area of 100 cells per experiment was also measured using the ImageJ software (NIH, *http://rsb.info.nih.gov/ij*).

Cell Migration Assays

In vitro directional cell migration assays were performed in modified Boyden chambers (NeuroProbe Inc., Bethesda, MD) as previously described,[30] except that migration was determined after 3 hours and that cells were stained with DAPI. Cell migration was quantified by counting the nuclei in at least ten random fields per filter under $\times$100 magnification. Nondirectional cell motility was measured by two-dimensional time-lapse video-microscopy. Cells were plated on laminin-111-coated wells and allowed to adhere for 2 hours. Metamorph imaging software was used to capture images every 5 minutes for 2 hours. A video was then constructed, and the migration of single cells was tracked using the Metamorph tracking function.

Cell Invasion Assay

Cell invasion assay was performed using 24-well Transwell inserts with 8-μm pore size (Millipore) coated with a thin layer of Matrigel (1.5 μg/mm^2). Cells (25,000 cells in 200 μl serum-free medium) were seeded on the upper chamber and the lower compartment was filled with 800 μl of serum-free medium. After 24 hours at 37°C, noninvading cells were removed by wiping the upper side of the membrane. Invading cells were fixed, stained, and counted as described for Boyden migration assay.

Cell Proliferation and Cell Death Assays

For the proliferation assays, cells harvested from subconfluent monolayers were seeded at 15,000/cm^2 in microtiter plates coated with 10 μg/ml laminin-111 and cultured 24 hours under standard conditions. Standard medium was then replaced by medium containing 1% serum and cell growth was monitored daily using MTT assay according to the manufacturer's instructions. The proliferation was confirmed by 5-bromo-2′-deoxyuridine incorporation into DNA. Proliferating cell nuclei were stained by alkaline phosphatase activity using a cell proliferation kit (Roche Applied Science, France) according to the manufacturer's instructions.

For cell survival, cells were cultured in standard conditions until confluence in microtiter plates. The standard medium was then replaced by serum-free medium and the surviving cells were quantified daily by the MTT assay. Apoptosis assays were performed using the same protocol, except that cells were fixed daily by 1% glutaraldehyde and nuclei were stained by DAPI (0.1 μg/ml). Nuclei were considered to have the normal morphology when glowing bright and homogenously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a totally fragmented morphology of nuclear bodies. The number of apoptotic cells was quantified by counting the apoptotic nuclei in five random fields per well under $\times$ 25 objective.

In Vivo *Tumorigenicity Studies and Immunohistochemical Analyses*

Animal work was performed in the animal facility of the school of medicine in accordance with institutional guidelines. In subcutaneous (s.c.) injection, exponentially growing cultures of control (mock-U87) and α6-expressing (α6-U87) cells were harvested and washed with serum-free medium. Cell suspension (1.2×10^6 cells/200 μl) was inoculated in the left (mock-U87) or right (α6-U87) flank of nude mice (5-week-old females from Harlan, Gannat, France). Tumor measures were taken weekly with calipers and volume was calculated as $(\text{width})^2 \times (\text{length}) \times (\pi/6)$ according to Osborne et al.[31] At the end of the experiment, mice were sacrificed and tumors surgically harvested, measured, and fixed in formalin. In intracerebral injections (i.c.), 10^5 cells were stereotactically injected into the frontal lobe of 4-week-old male balb/c nude mice (Charles River Laboratories, L'Arbresle, France). Mice weight was measured twice a week and animals were sacrificed at day 23, when the majority of the mice have lost more than 5g. Brains were frozen and horizontally sectioned. Areas of tumors in sections were measured using ImageJ software and tumor volumes were calculated as for s.c. tumors.

For histochemical and immunohistochemical analyses, paraffin-embedded 7-μm (s.c. tumors) or frozen 20-μm (i.c. tumors) sections were stained with H&E or immunolabeled using antibodies to factor VIII-related antigen (1/100), to laminin α1 chain (1/500), or to Ki-67 nuclear antigen (1/50). Colors were developed using Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and immunofluorescence was revealed using AlexaFluor-conjugated secondary antibodies. Terminal dUTP nick-end labeling (TUNEL) assay was performed using Apoptotag Red *in situ* apoptosis kit from Millipore (Saint Quentin en Yvelines, France).

Table 1. Cell Surface Expression of Integrin Subunits in Glioblastoma Cell Lines

Cell line	U87	U251	U138	SF763	SF767
Nonspecific	4.07	12.31	10.59	7.27	4.6
a6 subunit	4.76 (0.28%)	27.8 (95.0%)	46.4 (78.4%)	50.5 (90.1%)	36.4 (93.6%)
β1 subunit	239.8 (93.9%)	343.9 (98.3%)	525.7 (94.4%)	171.7 (85.2%)	137.2 (97.2%)
β4 subunit	4.52 (0.56%)	50.1 (72.1%)	53.1 (75.3%)	67.99 (88.3%)	82.3 (98.1%)

Flow cytometry analysis was performed using mAb directed towards integrin subunits on glioblastoma cell lines as described in the experimental section. Results, from a representative experiment, are expressed as means in fluorescence intensity (arbitrary units). The percentage of cells expressing each integrin subunit is indicated in brackets.

Statistical Analysis

Data shown are means ± SEM for at least three separate experiments ($n \geq 3$). Statistical differences were analyzed by use of either Student's *t*-test for paired data or the Wilcoxon rank sum test. A P value <0.05 was considered significant.

Results

Stable Expression of Functional α6β1 Integrin in U87 Cell Line

Several arguments, including the fact that integrin α6β1 is overexpressed in glioblastoma tissue, while weakly expressed in normal brain,[20] suggest that this integrin might be involved in glioma progression. To address this question, we first analyzed the cell surface expression of this integrin by FACS analysis on five glioblastoma cell lines in culture (U87, U251, U138, SF763, SF767). As shown in Table 1, among the five malignant glioma cells tested, only the U87 cell line was negative for α6 subunit expression at cell surface. To form a functional receptor the α6 subunit should be associated either with β1 or β4 subunits. All five glioma cell types, including the U87 cell line, expressed high levels of the β1 integrin subunit, whereas β4 displayed the same expression pattern as α6, with U87 cell being the only β4-negative cell line (Table 1).

We therefore used the U87 cell line to study the role of α6β1 integrin in malignant glioma cells. For this purpose, we stably expressed human α6 subunit in the U87 cell line via retroviral-mediated gene transfer. The U87 parental cell line was infected with retroviral particles carrying either an empty vector (mock-U87) or a vector including the cDNA encoding for α6 (α6-U87). Mock-U87 and α6-U87 cells were then selected by FACS. As expected, α6-U87 cells expressed the α6 integrin subunit, whereas mock-U87 were negative (Figure 1A). Expression of α6 in U87 cells did not modify the expression of the other integrins (data not shown). Particularly, the expression of β1 and β4, the partners of α6, remained unchanged (Figure 1A).

Because α6-U87 cells do not express the β4 subunit, it should be expected that α6 is associated with β1 in these cells. This was confirmed by co-immunoprecipitation experiments. Indeed, as illustrated in Figure 1B, an antibody against α6 was able to co-immunoprecipitate the β1 subunit from α6-U87 cell lysates, while no co-immunoprecipitation was observed with mock-U87 cells. In the same way, the anti-β1 antibodies co-immunoprecipitated α6 only when using α6-U87 cell lysates.

To know whether the integrin α6β1 expressed at α6-U87 cell surface was functional, we first performed adhesion and spreading assays. As seen in Figure 1C, α6-U87 cells adhered more readily to laminin-111 than mock-U87 cells. Besides, function-blocking antibodies against α6 and β1 subunits, but not against α3 or β4 subunits, inhibited adhesion to laminin-111 of α6-U87 and the other α6-expressing glioblastoma cells, suggesting that, when expressed, α6β1 is the main laminin-111 receptor in glioblastoma cells (Figure 1D). Cell spreading on laminin-111 was also facilitated by the expression of α6β1 (Figure 1E). One hour after plating, mock-U87 cells appeared rounded or slightly spread, while α6-U87 cells displayed large lamellipodia. Quantification of cell surfaces with the Metamorph software gave a mean of 542 ± 44 μm^2 and 939 ± 68 μm^2 for mock-U87 and α6-U87 cells, respectively. The percentage of spread cells on laminin-111 was 35.2 ± 1.7% and 76.7 ± 2.5% for mock-U87 and α6-U87 cells, respectively. All these differences of behavior on laminin-111 between mock-U87 and α6-U87 cells are likely due to the expression of α6β1. Indeed, no difference could be observed when type I collagen, which is not recognized by α6β1, was used as a matrix for adhesion (Figure 1C) or spreading (Figure 1E) assays.

α6β1 Expression Enhances Glioblastoma Cells Growth and Decreases Apoptosis in Vitro

It is well established that cell−ECM interactions mediated by integrins are necessary for cell cycle progression and cell proliferation.[32] To investigate whether α6β1 is involved in glioma cell proliferation, the two U87 variants were grown on laminin-111-coated dishes in Eagle's minimal essential medium supplemented with 1% fetal bovine serum and cell growth was monitored by MTT assay. In these conditions, α6-U87 cells grew faster than mock-U87 cells (Figure 2A). Growth curves showed an increased lag phase duration in the case of mock-U87 cells. To confirm that the reduced cell number observed at early time periods was really due to growth inhibition, the proliferation was visualized by detecting cells that have incorporated 5-bromo-2′-deoxyuridine into DNA. As shown in Figure 2B, less nuclei exhibited 5-bromo-2′-deoxyuridine uptake in mock-U87 cells

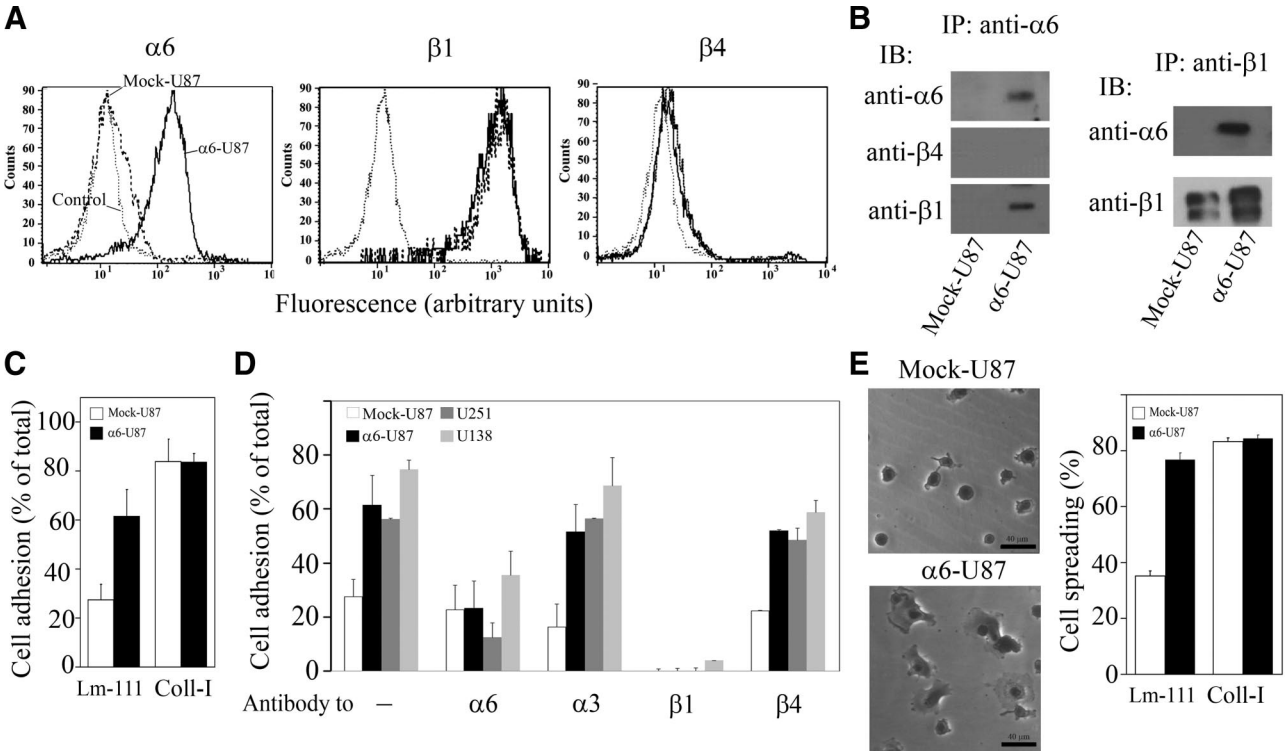

Figure 1. Cell surface expression and function of the integrin $\alpha6\beta1$. **A:** Mock-U87 and $\alpha6$-U87 cells were resuspended in the presence of mAbs to $\alpha6$, $\beta1$ and $\beta4$ integrin subunits for 60 minutes at 4°C. After staining with AlexaFluor−647-conjugated secondary antibody, cell-bound fluorescence was quantified by flow cytometric analysis. **B:** Cell lysates from U87 cell variants were incubated with mAbs to $\alpha6$ or $\beta1$ integrin subunits. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and probed with indicated polyclonal antibodies. IP: immunoprecipitation, IB: immunoblot. $\alpha6$: $Mr = 150$, $\beta4$: $Mr = 190$, $\beta1$: $Mr = 110$. **C:** Mock-U87 (white bars) and $\alpha6$-U87 (black bars) cells were plated in 96-well microtiter plates coated with 10 μg/ml laminin-111 (Lm-111) or type I collagen (Coll-I) and allowed to adhere for 30 minutes at 37°C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. **D:** U87 variants and two other glioma cell lines (U251 and U138) were pre-incubated with 10 μg/ml of function-blocking mAbs against $\alpha6$, $\alpha3$, $\beta1$, or $\beta4$ integrin subunits for 30 minutes at room temperature and adhesion was assessed on laminin-111. **E:** Mock-U87 (white bars) and $\alpha6$-U87 (black bars) cells were plated in 24-well plates coated with 10 μg/ml laminin-111 or type I collagen for 1 hour at 37°C. After washing, cells were fixed and stained with crystal violet. Representative pictures are shown in left panels. The percentage of spread cells was determined as described in the experimental section (right panel). Scale bar = 40 μm. All data shown are means (± SEM) from at least three experiments performed in triplicate.

than in $\alpha6$-U87 cells, suggesting that $\alpha6$ expression affected DNA synthesis.

Hypoxia and nutrient deprivation are physiological stresses associated with tumor progression. In breast cancer, it has been described that $\alpha6\beta1$ can protect cells from apoptosis induced by nutrient-poor conditions together with hypoxia *in vitro*.[33] To investigate the survival function of $\alpha6\beta1$ in glioblastoma, we examined the effect of $\alpha6$ expression on survival of U87 cell variants in serum-deprived conditions. Confluent cell monolayers were cultured in serum-free medium and cell survival was assessed using the MTT assay. Both U87 cell variants grew similarly for 2 days after serum withdrawal. Four days after serum deprivation, we observed a decrease in mock-U87 cell viability, whereas no such difference was found with $\alpha6$-U87 cells (Figure 2C). After 5 days, cell viability was 43.3 ± 3.6% and 74 ± 3.5% for mock-U87 and $\alpha6$-U87 cells respectively.

To test whether the mock-U87 cell death induced by serum withdrawal was due to apoptosis, we evaluated the percentage of apoptotic cells by nuclear morphology of DAPI-stained cells. Cells with condensed, fragmented chromatin were scored as apoptotic. Four days after serum withdrawal, we observed numerous fragmented nuclei for mock-U87 cells, whereas very few apoptotic $\alpha6$-U87 cells could be detected (Figure 2D). Quantification of apoptosis showed that about 20% of mock-U87 cells were apoptotic as soon as day 3 and this percentage increased with time, reaching 60.8 ± 6.4% on day 5 (Figure 2E). In the case of $\alpha6$-U87 cells, fragmented nuclei could hardly be seen before day 5, with only 11.2 ± 2.7% of apoptotic cells at this time. These results are in accordance with the cell survival measured by MTT. Altogether, these data suggest that expression of $\alpha6\beta1$ delayed glioma cell death and protected them from apoptosis in nutrient-poor conditions.

$\alpha6\beta1$ Is Essential for Glioma Cells Migration and Invasion

One of the particularities of glioblastoma, that prevents successful surgical treatment, is linked to their ability to infiltrate the whole brain by single-cell migration to form new tumor foci. Deposits of laminin-111 have been found in the border zone between the normal brain and the infiltrating glioma cells.[34,35] We thus first investigated the effect of $\alpha6$ expression on laminin-mediated cell motility

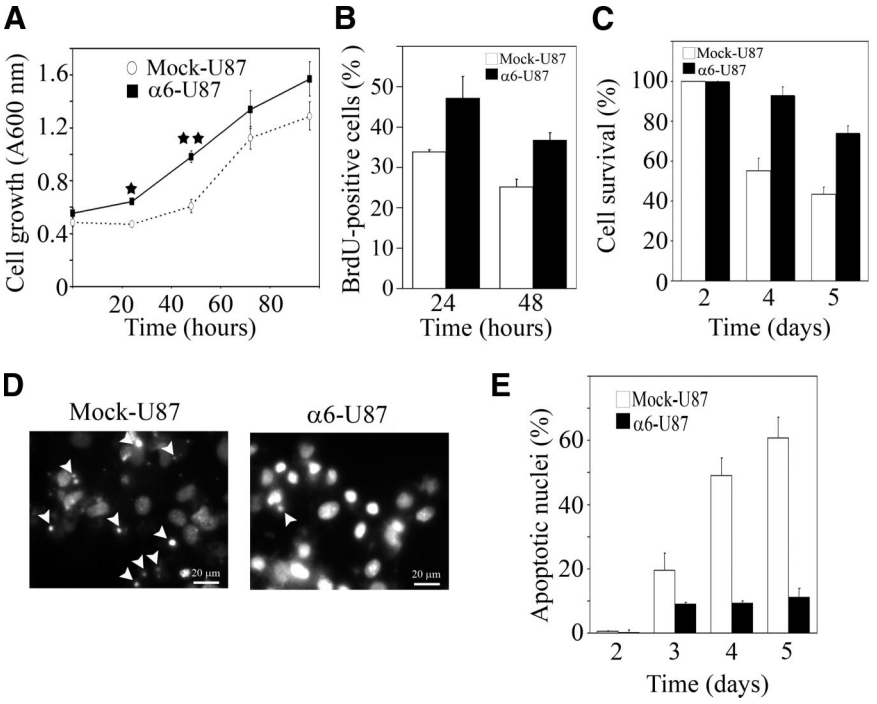

Figure 2. Expression of α6β1 promotes glioblastoma cells proliferation and survival *in vitro*. **A:** Mock-U87 (white circles) and α6-U87 (black squares) cells were cultured with 1% serum for the indicated periods of time in 96-well microtiter plates coated with 10 μg/ml laminin-111 and cell growth was evaluated using MTT assay. Statistically significant differences $^{*}P < 0.05$ and $^{**}P < 0.01$ are indicated. **B:** Cells were cultured for 24 or 48 hours in 24-well microtiter plates coated with 10 μg/ml laminin-111. The percentage of proliferating mock-U87 (white bars) and α6-U87 (black bars) cells was assessed by 5-bromo-2′-deoxyuridine incorporation assay. **C:** Mock-U87 (white bars) and α6-U87 (black bars) cells were cultured in 96-well microtiter plates. Cells were monitored for their survival at daily intervals on serum removal using MTT assay. **D:** Four days after serum withdrawal, cells were stained with DAPI and visualized by fluorescence microscopy. Cells with fragmented nuclei or condensed chromatin (**arrowheads**) were referred to as apoptotic. Scale bar = 20 μm. **E:** The percentage of apoptotic nuclei induced by serum deprivation in mock-U87 (white bars) and α6-U87 (black bars) cells was quantified at different times. All data represent the mean ± SEM of three separate experiments performed in triplicate.

of malignant glioma by two different approaches: two-dimensional, time-lapse video-microscopy to measure random (or non-directed) movement and modified Boyden chambers to quantify directed migration toward an ECM gradient. Cells were plated on laminin-111 and the movement of individual cells (100 cells of each U87 variant) was followed over a period of 2 hours by time-lapse video-microscopy (Figure 3A). Quantification of velocity clearly showed that α6-U87 cells had a higher motility rate (58.9 μm/h) than control cells (26.6 μm/h). Moreover, when cell migration paths from time-lapse recordings were reported on a graph (origins set to x = 0, y = 0), it becomes obvious that mock-U87 cells were unable to migrate persistently toward a given direction and stayed near their starting point. Conversely, α6-U87 cells migrated in different directions over the entire space (Figure 3A, right panels). Moreover, this migration of α6-U87 cells on laminin-111 was dependent on α6β1. Indeed, function-blocking mAbs against α6 and β1, but not against α3, blocked motility of α6β1-expressing cells to the level of mock-U87 cells (Figure 3B). Similar results were obtained with modified Boyden chambers, α6-U87 cells migrating faster to laminin-111 than mock-U87 cells (Figure 3C). In addition, no differences in cell migration could be observed with type I collagen, confirming that the differences in the migratory behavior between U87 variants are likely due to the cell surface expression of integrin α6β1.

We then used *in vitro* Matrigel invasion assays to determine whether the increased migration rate of α6-U87 cells was associated with invasive properties. This is clearly the case, as, in the absence of serum, mock-U87 cells did not invade at all Matrigel, whereas α6-U87 cells were highly invasive (Figure 3D). To know whether the enhanced invasiveness was due to α6β1 activity we repeated the experience in the presence of blocking antibodies directed against integrin subunits. As shown in Figure 3E, antibodies to α6 and β1 subunits completely abolished Matrigel invasion by α6-U87 cells, while anti-

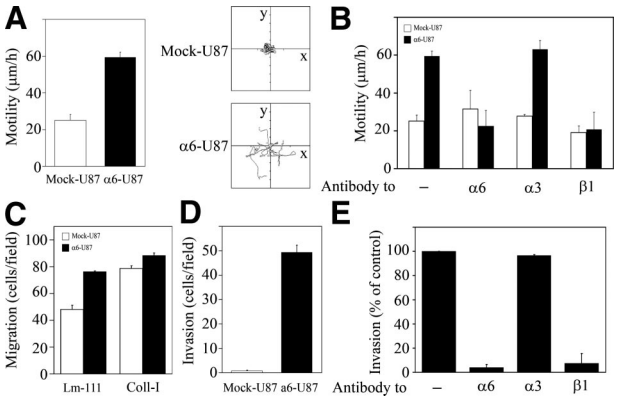

Figure 3. Expression of α6β1 enhances glioblastoma cells migration and invasion *in vitro*. **A:** Mock-U87 and α6-U87 cells were plated on 24-well culture plates precoated with 10 μg/ml laminin-111, and the nondirected migration of individual cells (100 cells for each U87 variant) was recorded by time-lapse video-microscopy over 2 hours at 37°C. Representative migration paths of five cells are reported in the right panels using position parameters. **B:** Nondirected migration of mock-U87 (white bars) and α6-U87 (black bars) was recorded as above after pre-incubation for 30 minutes at room temperature with 10 μg/ml of function-blocking mAbs against α6, α3, or β1 integrin subunits. Data shown are the mean of a representative experiment from three performed in duplicate. **C:** Directed cell migration was measured in modified Boyden chambers using porous membrane precoated with 10 μg/ml laminin-111 (Lm-111) or type I collagen (coll-I). Mock-U87 (white bars) and α6-U87 (black bars) cells were seeded into the upper reservoir and allowed to migrate through the filter toward the lower reservoir for 3 hours at 37°C. Cells that migrated to the underside of the filter were stained with DAPI and counted. **D:** Mock-U87 and α6-U87 cells were plated in the upper reservoir of Transwell chambers containing a thin layer of Matrigel. After 24 hours at 37°C, cells that migrated through the filter were stained and counted. **E:** Matrigel invasion by α6-U87 cells was quantified after pre-incubation with 10 μg/ml of function-blocking mAbs against α6, α3, or β1 integrin subunits for 30 minutes at room temperature. All data shown are means (± SEM) from 3 or 4 experiments performed in triplicate.

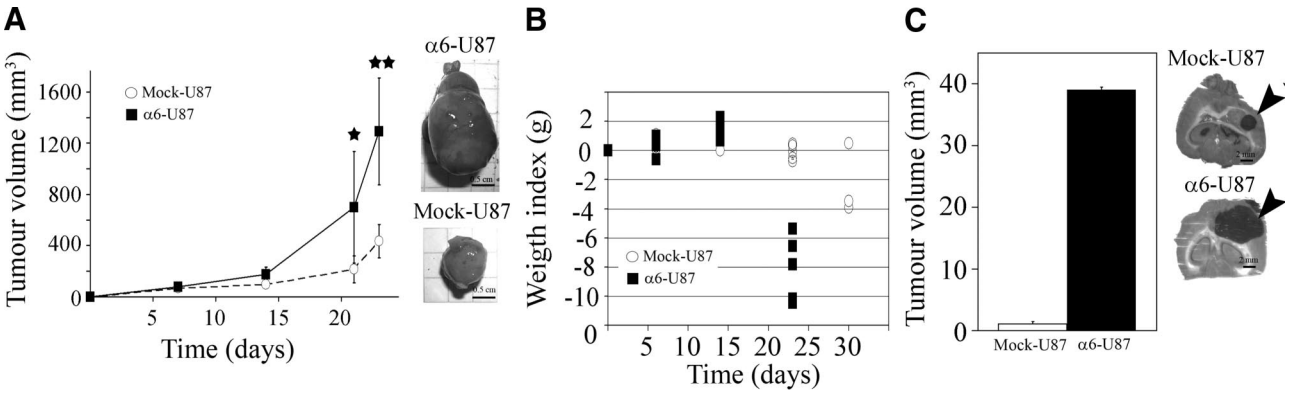

Figure 4. Expression of $\alpha 6\beta 1$ enhances tumor growth *in vivo*. **A:** Subcutaneous implantation of mock-U87 (white circles) and α6-U87 (black squares) cells in nude mice ($n = 15$ per group). Tumor size was measured weekly and data ($\pm$ SEM) were analyzed using the Wilcoxon rank sum test. Statistically significant differences are indicated: $^{*}P < 0.05$ and $^{**}P < 0.02$ ($n = 15$). Representative s.c. tumors after surgical resection are shown in the right. **B:** Mock-U87 (white circles) and α6-U87 (black squares) cells were injected in the frontal lobe of nude mice brain and mice weight was monitored twice a week. **C:** At day 23 postinoculation, mice were sacrificed and tumor size was measured. Tumor volumes are means $\pm$ SEM ($n = 4$ for Mock-U87, $n = 5$ for α6-U87). Representative sections corresponding to the center of i.c. tumors are shown in the right. **Arrowheads** indicate the tumors.

bodies against $\alpha 3$ or $\beta 4$ subunits did not display any effect. Identical results were observed with the two invading glioblastoma cells U251 and U138 (see supplemental Figure S1 at *http://ajp.amjpathol.org*), confirming that the invading phenotype depended on the integrin $\alpha 6\beta 1$ rather than integrin $\alpha 6\beta 4$. The above results show that expression of $\alpha 6\beta 1$ dramatically enhanced the migratory and invasive potential of U87 cells *in vitro*.

α6β1 Expression Promotes in Vivo *Tumor Growth*

To determine whether increased mobility and invasiveness due to integrin $\alpha 6\beta 1$ expression could lead to an enhanced *in vivo* aggressiveness, we first analyzed the ability of cells to develop tumors in nude mice. Each animal was inoculated s.c. with 1.2×10^6 mock-U87 cells in one flank and 1.2×10^6 α6-U87 cells in the other flank and tumors size was periodically measured. In the flank injected with α6-U87 cells, tumor growth was strongly enhanced compared with the opposite side of the animals inoculated with mock-U87 cells (Figure 4A). At 23 days postinoculation, the tumor volume that developed from α6-U87 cells was about threefold higher than that calculated for tumors originating from mock-U87 cells (1293 ± 417 mm^3 *vs.* 435 ± 130 mm^3, respectively; $P > 0.02$) (Figure 4A).

To examine whether α6 expression could alter U87 cells tumorigenicity in the central nervous system, mature male nude mice were intracerebrally (i.c.) injected with either mock-U87 or α6-U87 cells (10^5 cells per mouse). Mice were regularly examined to detect clinical symptoms of deterioration due to tumor progression, such as weight loss. As illustrated in Figure 4B, all α6-U87 cell-inoculated mice presented a rapid loss of weight ranging from 5 to 10 g and had to be sacrificed at day 23 postinoculation. Conversely, in the case of mock-U87 cell-inoculated animals, a less pronounced weight loss was only observed from day 30. To compare tumor volumes, 4 mice bearing mock-U87 cell-induced tumors were sacrificed in parallel with animals bearing α6-U87 cell-induced tumors. We observed a 39-fold increase in the average tumor volume for α6-U87 tumors compared with mock-U87 tumors (Figure 4C). It thus appears that the expression of the integrin $\alpha 6\beta 1$ by U87 glioblastoma cells lead to an even steeper increase in tumor growth when injected in the more physiological environment of brain.

α6β1 Expression Modifies Tumor Cell Morphology and Vasculature

To study the possible mechanisms causing enhanced tumor growth, we examined the histological features of tumors arising from our U87 cell variants. Beside the obvious difference in the i.c. tumors size, a striking finding was the presence of infiltrative foci in the margin of α6-U87 cell-induced tumors that were not found in mock-U87 cell-induced tumors (Figure 5A, left panels). Among the five analyzed α6-U87 cell-induced tumors, four displayed infiltrative foci (mean of 2.5 per tissue section), while none of the four analyzed control tumors did. Moreover, α6-U87 tumors presented more intense staining and cell density and more irregular boundaries than control tumors. Cells also displayed an intriguing alteration of morphology; cells from mock-U87-induced tumors were fusiform, while cells from α6-U87-induced tumors displayed a rounded shape (not shown).

Gliomas are among the most angiogenic and highly vascularized of human tumors.[36] We therefore compared the level of neovascularization of U87 cell-induced tumors by immunostaining of coagulation factor VIII. In i.c. experiments, α6-U87 tumors possessed longer and more organized vessels than mock-U87 tumors (Figure 5A, middle panels). The quantitative analysis of 10 microscopic fields of i.c tumors sections indicates that the total vessel area is twofold higher in α6-U87 tumors compared with mock-U87 tumors (Figure 5B, left). Moreover, α6 expression was accompanied by an increase of microvascular density from 11.1 ± 1.7 vessels per field for mock-U87 tumors to 25.7 ± 2.5 vessels per field for α6-U87 tumors (Figure 5B, right). The difference in the

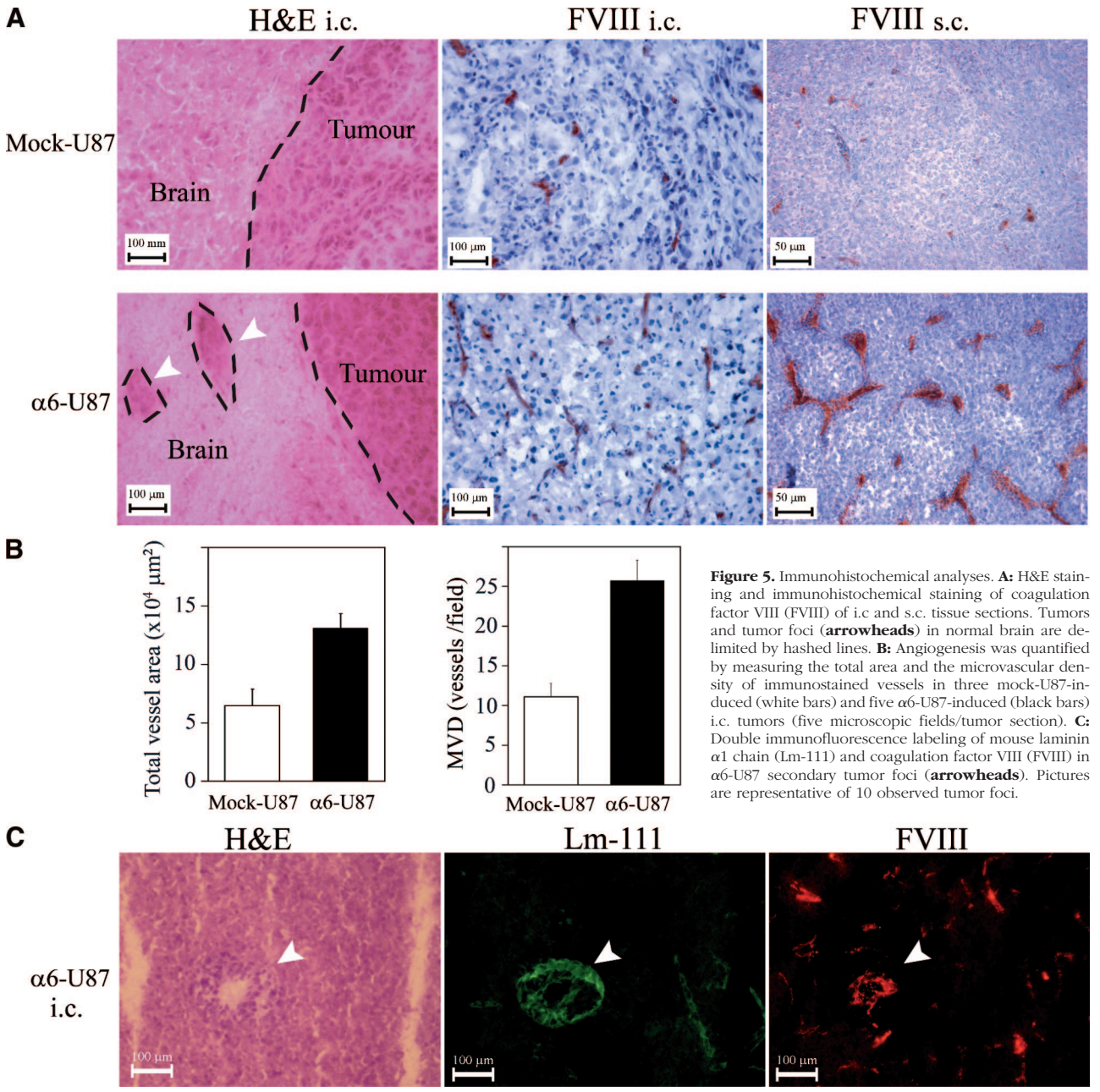

Figure 5. Immunohistochemical analyses. **A:** H&E staining and immunohistochemical staining of coagulation factor VIII (FVIII) of i.c and s.c. tissue sections. Tumors and tumor foci (**arrowheads**) in normal brain are delimited by hashed lines. **B:** Angiogenesis was quantified by measuring the total area and the microvascular density of immunostained vessels in three mock-U87-induced (white bars) and five α6-U87-induced (black bars) i.c. tumors (five microscopic fields/tumor section). **C:** Double immunofluorescence labeling of mouse laminin α1 chain (Lm-111) and coagulation factor VIII (FVIII) in α6-U87 secondary tumor foci (**arrowheads**). Pictures are representative of 10 observed tumor foci.

tumor vasculature was even more obvious in s.c. experiments, with α6-U87 tumors displaying large sinuous vessels with numerous capillary sprouts (Figure 5A, right panels). It thus appears that the increased growing of α6-U87 tumors is associated with a well-developed vasculature.

To establish a relationship between the expression of α6β1 integrin and the presence of infiltrative foci in α6-U87 tumors, we performed double immunofluorescence labeling, using the 1057+ Ab, specific for mouse laminin α1 chain, and an anti-coagulation factor VIII Ab. As illustrated by the representative example shown in Figure 5C, all of the infiltrative foci observed ($n = 10$) were associated with the presence of mouse laminin immunolabeling. Moreover, a labeling of coagulation factor VIII was also detected in all of the foci, suggesting that the invasive glioblastoma cells developed around blood vessels.

α6β1 Enhances Proliferation and Decreases Apoptosis in Vivo

The rate of tumor growth depends on the balance between cell proliferation and cell death. The above results show that α6 modulates glioblastoma cell growth and apoptosis *in vitro*. To test whether α6 also acts on glioblastoma cell proliferation and survival *in vivo*, tumor tissue sections were subjected to Ki-67 immunohistochemical staining to analyze proliferating cells and to TUNEL assay to detect apoptotic cells. Ki-67 staining of

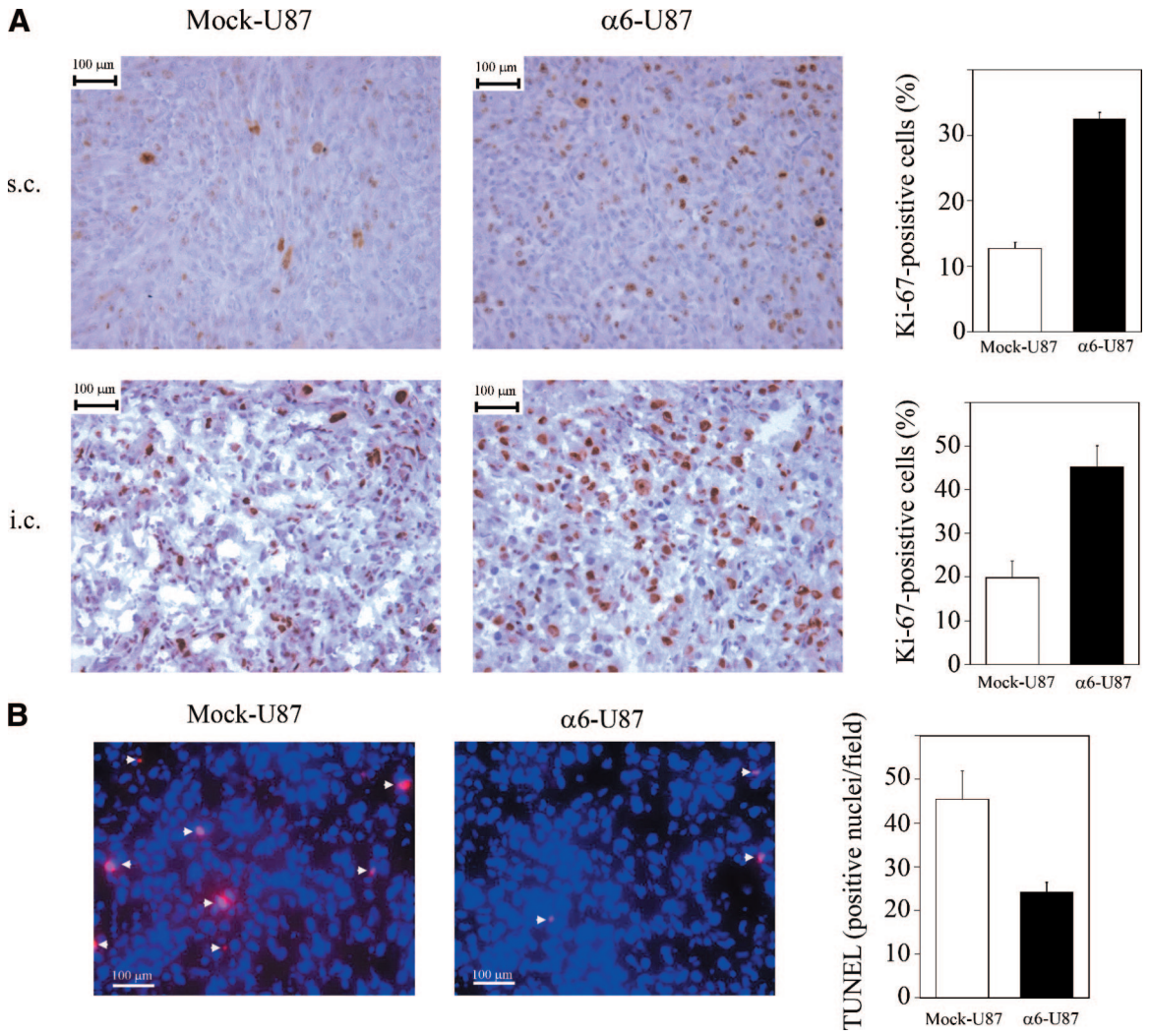

Figure 6. Expression of $\alpha 6\beta 1$ promotes tumor cells proliferation and survival *in vivo*. **A:** Serial sections of i.c and s.c. tumors were scored for proliferation by immunohistochemical staining of the Ki-67 nuclear antigen (**left**). Quantification of proliferation (**right**) was performed in three mock-U87- and five α6-U87-induced tumors (five microscopic fields/tumor section). Cells were considered Ki-67-positive and referred to as in the proliferative state when a dark brown staining of the nucleus was observed. **B:** TUNEL assay performed i.c tumors sections. Cells were considered TUNEL-positive (**arrowheads**) and referred to as in the apoptotic state when red nuclei, corresponding to a marked DNA degradation, were observed. Areas of obvious necrosis were excluded from counting (**left**). Quantification of apoptosis (**right**) was performed in three mock-U87- and five α6-U87-induced tumors (five microscopic fields/tumor section).

s.c. tumors showed significant differences, with a 2.5-fold increase in Ki-67-positives cells for α6-U87 tumors, as compared with mock-U87 tumors (Figure 6A, upper panels). A difference, albeit of smaller magnitude, was also observed at the tumor periphery, where the proliferation index was at its maximum for both tumors, with an average of 110.2 ± 5.2 and 129.6 ± 8.1 cells per field for mock-U87 and α6-U87 tumors, respectively (data not shown). In the i.c. tumors, we did not observe any difference between the periphery and the center of tumors, but we confirmed that α6-U87 tumors display a more proliferating activity than mock-U87 tumors with, respectively, 45.2 ± 4.9% and 19.9 ± 3.8% of Ki-67-positive cells (Figure 6A, lower panels).

TUNEL assays revealed no labeling in normal brain tissue bordering α6-U87 and mock-U87 tumors (data not shown), while both cell lines developed tumors displaying apoptotic cells (Figure 6B). Quantification of TUNEL labeling revealed that mock-U87 tumors had higher rate of apoptosis compared with α6-U87 tumors (45.5 ± 6.5 *vs.* 24.1 ± 2.3 cells per field). Altogether, these results suggest that difference in the growth rate of tumors induced by the two U87 cells may partly rely on increased proliferation and decreased apoptosis as observed *in vitro*.

Discussion

The data presented here demonstrate for the first time that the laminin receptor $\alpha 6\beta 1$ plays an essential role in glioblastoma progression. This was suggested by previous studies showing that α6 integrin was strongly expressed in glioblastoma tissue, as compared with normal brain.[20] This observation was confirmed and extended to other central nervous system tumors (astrocytoma, meningioma, and neuroblastoma).[21] In addition, as reported in this study and by others,[11,22] glioblastoma cell lines

express α6 integrin subunit. The only exception is, to our knowledge, the U87 cell line. We thus stably expressed the integrin α6β1 in U87 cells to study its role in the malignancy of glioblastoma cells. We show in this study that cell surface expression of the α6β1 integrin led to dramatic changes in the behavior of tumor U87 cell, both *in vitro* and *in vivo*.

Nude mice receiving either subcutaneous or intracerebral inoculation of α6-U87 cells developed substantially more voluminous tumors than mice injected with control mock-U87 cells. This is in accordance with previous studies demonstrating that blocking of α6β1 integrin with a specific antibody or impeding its expression markedly suppress breast carcinoma growth *in vivo* and abolish metastatic colonization of lungs.[17,19] As other integrins, the α6 integrin (primarily α6β4) has been largely involved in regulation of cell proliferation and survival.[37] Moreover, α6β1 protects cells from apoptosis induced by serum deprivation together with hypoxia and facilitates tumor cell survival in distant organs.[17,19] Therefore, the difference in tumor growth we observed between α6-U87 and mock-U87 cells was likely partly related to changes in the balance between cell proliferation and survival. Indeed, expression of α6β1 enhanced proliferation and decreased apoptosis of U87 cells both in the tumor and when plated on laminin.

The development of new blood vessels is essential for local tumor progression. This is particularly true in the case of glioblastoma, characterized by exuberant angiogenesis and by newly formed vessels that are structurally and functionally abnormal (for a review, see Jain et al.[38]). We show here that a striking difference between α6-U87 cell- and mock-U87 cell-induced tumors (especially in s.c. tumors) is a marked increase in vascularization. Moreover, α6-U87 tumors displayed large and sinuous vessels with numerous capillary sprouts. The increase in the tumor vasculature is associated with the presence of infiltrative foci in the case of α6-U87 cell-induced tumors. All this is in agreement with a correlation between biological aggressiveness and degree of malignancy in gliomas.[39] Thus, the expression of α6β1 integrin by glioblastoma cells could contribute to tumor growth by facilitating nutrient availability, but also to tumor cell invasiveness.

The poor prognosis of glioblastoma is due to their highly invasive nature. Glioblastoma, like other tumors of the central nervous system, exhibit more local infiltration and rare metastasis, as compared with cancers of ectodermal origin.[2] After surgical resection of the primary tumor mass, individual glioblastoma cells can migrate into the surrounding normal brain tissue and form numerous microscopic satellites that escape adjuvant radiation and chemotherapy and eventually lead to regrowth of a recurrent tumor and to patient death.[40] Consequently, understanding the mechanisms of glioma invasion is essential to improve the efficacy of oncologic treatments. In the present study, we show that mock-U87 cell-induced tumors displayed well-delimited boundaries when compared with α6-U87 cells. Besides, some foci of tumor cells were observed near the tumor mass, exclusively in the case of α6β1-expressing U87 cells. This invasive behavior was confirmed *in vitro*, as expression of integrin α6β1 is required for Matrigel invasion by U87 cells. The expression of integrin α6β1 appears to confer the invasive phenotype in melanoma,[41] and in prostate[42,43] and breast[18] cancers. The invasive potential of glioblastoma cells is dependent on α6β1 ligation, as function-blocking antibodies against α6 and β1 subunits, but not against α3 or β4 subunits, inhibited Matrigel invasion by α6-U87 cells, as well as by U251 and U138 glioblastoma cell lines (Figure 3 and supplemental Figure S1 at *http://ajp.amjpathol.org*). However, SF763 and SF767 cells, which also display α6 at their cell surface, were unable to invade Matrigel (data not shown), indicating that, although the integrin is necessary, other molecules or signals are required for the cell to acquire an invasive behavior.

Matrix metalloproteinases (MMPs) have been implicated in glioma invasion and angiogenesis by facilitating extracellular matrix degradation. Glioma cells produced MMP-9 and MMP-2, and the corresponding mRNA and protein levels are found to be higher in tumor than in normal brain.[44] Moreover, a correlation was found between MMP expression and the degree of malignancy of human brain tumors including glioblastoma.[45] During the present study, we observed, by zymographic analysis, that U87 cells secrete both MMP-2 and MMP-9 (data not shown). However, in contrast to the result from adenoid cystic carcinoma,[46] the expression of the integrin α6β1 did not affected MMP secretion (data not shown).

Glioblastoma malignant cells diffuse in normal brain along certain preferred paths, following myelinated axons or the basement membrane of blood vessels.[47,48] It is known that in normal brain tissue, laminin is associated with blood vessels and is overexpressed when brain is confronted to glioma cells.[22] In addition, *in vitro* studies have demonstrated that laminin-111 is one of the most permissive substrates for adhesion and migration of glioma cells.[25–27] Using both time-lapse video-microscopy and haptotaxis assays, we show here that expression of the integrin α6β1 noticeably enhanced glioblastoma cells migration on laminin-111. Besides, we observed in the present study that all of the infiltrative foci found near the tumors induced by α6β1-expressing cells are associated with a labeling of both laminin-111 and anti-coagulation factor VIII. Thus, it can be postulated that laminin-111 may guide tumor cell migration and invasion of normal brain and that the expression of integrin α6β1, along with other partners,[49] facilitates this process.

Our current working hypothesis is that the expression of integrin α6β1 and the production of laminin in glioblastoma contribute both to tumor growth by favoring tumor cell proliferation and survival and to the invasive phenotype of tumor cells. Invasion could occur on the surfaces of laminin-coated structures such as the blood vessels and account for the known patterns of glioma progression. Blockage of the expression or function of α6β1 or laminin would be an essential step in limiting tumor growth and in confining the malignant cells to tumor core to allow effective surgical resection.

Acknowledgments

The authors thank Dr. Arnould Sonnenberg (Cancer Institute, Amsterdam, The Netherlands) for his gift of the human α6 integrin cDNA, Dr. Michal Stachowiak for the glioblastoma cell lines, and Dr. Takako Sasaki (Martinsried, Germany) for 1057+ antibody. We thank Charles Prévôt and Jeannine Secchi for their technical assistance, Oulfat Amin Ali for her help, and Dr. Imad About and Dr. Patricia Simon-Assmann for their critical comments on the manuscript.

References

1. Ohgaki H, Kleihues P: Epidemiology and etiology of gliomas. Acta Neuropathol 2005, 109:93–108
2. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P: The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007, 114:97–109
3. Figarella-Branger D, Colin C, Coulibaly B, Quilichini B, Maues De Paula A, Fernandez C, Bouvier C: [Histological and molecular classification of gliomas]. Rev Neurol (Paris) 2008, 164:505–515
4. Takada Y, Ye X, Simon S: The integrins. Genome Biol 2007, 8:215
5. Bellail AC, Hunter SB, Brat DJ, Tan C, Van Meir EG: Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. Int J Biochem Cell Biol 2004, 36:1046–1069
6. Bello L, Francolini M, Marthyn P, Zhang J, Carroll RS, Nikas DC, Strasser JF, Villani R, Cheresh DA, Black PM: Alpha(v)beta3 and alpha(v)beta5 integrin expression in glioma periphery. Neurosurgery 2001, 49:380–389; discussion 390
7. Bello L, Lucini V, Giussani C, Carrabba G, Pluderi M, Scaglione F, Tomei G, Villani R, Black PM, Bikfalvi A, Carroll RS: IS20I, a specific alphavbeta3 integrin inhibitor, reduces glioma growth in vivo. Neurosurgery 2003, 52:177–185; discussion 185–176
8. Yamada S, Bu XY, Khankaldyyan V, Gonzales-Gomez I, McComb JG, Laug WE: Effect of the angiogenesis inhibitor Cilengitide (EMD 121974) on glioblastoma growth in nude mice. Neurosurgery 2006, 59:1304–1312; discussion 1312
9. MacDonald TJ, Taga T, Shimada H, Tabrizi P, Zlokovic BV, Cheresh DA, Laug WE: Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery 2001, 48:151–157
10. Maglott A, Bartik P, Cosgun S, Klotz P, Ronde P, Fuhrmann G, Takeda K, Martin S, Dontenwill M: The small alpha5beta1 integrin antagonist. SJ749, reduces proliferation and clonogenicity of human astrocytoma cells. Cancer Res 2006, 66:6002–6007
11. Kawataki T, Yamane T, Naganuma H, Rousselle P, Anduren I, Tryggvason K, Patarroyo M: Laminin isoforms and their integrin receptors in glioma cell migration and invasiveness: Evidence for a role of alpha5-laminin(s) and alpha3beta1 integrin. Exp Cell Res 2007, 313:3819–3831
12. Jones JC, Kurpakus MA, Cooper HM, Quaranta V: A function for the integrin alpha 6 beta 4 in the hemidesmosome. Cell Regul 1991, 2:427–438
13. Torimura T, Ueno T, Kin M, Inuzuka S, Sugawara H, Tamaki S, Tsuji R, Sujaku K, Sata M, Tanikawa K: Coordinated expression of integrin alpha6beta1 and laminin in hepatocellular carcinoma. Hum Pathol 1997, 28:1131–1138
14. Begum NA, Mori M, Matsumata T, Takenaka K, Sugimachi K, Barnard GF: Differential display and integrin alpha 6 messenger RNA overexpression in hepatocellular carcinoma. Hepatology 1995, 22:1447–1455
15. Schmelz M, Cress AE, Scott KM, Burger F, Cui H, Sallam K, McDaniel KM, Dalkin BL, Nagle RB: Different phenotypes in human prostate cancer: alpha6 or alpha3 integrin in cell-extracellular adhesion sites. Neoplasia 2002, 4:243–254
16. Hangan D, Morris VL, Boeters L, von Ballestrem C, Uniyal S, Chan BM: An epitope on VLA-6 (alpha6beta1) integrin involved in migration but not adhesion is required for extravasation of murine melanoma B16F1 cells in liver. Cancer Res 1997, 57:3812–3817
17. Wewer UM, Shaw LM, Albrechtsen R, Mercurio AM: The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. Am J Pathol 1997, 151:1191–1198
18. Mukhopadhyay R, Theriault RL, Price JE: Increased levels of alpha6 integrins are associated with the metastatic phenotype of human breast cancer cells. Clin Exp Metastasis 1999, 17:325–332
19. Lee TH, Seng S, Li H, Kennel SJ, Avraham HK, Avraham S: Integrin regulation by vascular endothelial growth factor in human brain microvascular endothelial cells: role of alpha6beta1 integrin in angiogenesis. J Biol Chem 2006, 281:40450–40460
20. Gingras MC, Roussel E, Bruner JM, Branch CD, Moser RP: Comparison of cell adhesion molecule expression between glioblastoma multiforme and autologous normal brain tissue. J Neuroimmunol 1995, 57:143–153
21. Previtali S, Quattrini A, Nemni R, Truci G, Ducati A, Wrabetz L, Canal N: Alpha6 beta4 and alpha6 beta1 integrins in astrocytomas and other CNS tumors. J Neuropathol Exp Neurol 1996, 55:456–465
22. Knott JC, Mahesparan R, Garcia-Cabrera I, Bolge Tysnes B, Edvardsen K, Ness GO, Mork S, Lund-Johansen M, Bjerkvig R: Stimulation of extracellular matrix components in the normal brain by invading glioma cells. Int J Cancer 1998, 75:864–872
23. Belot N, Rorive S, Doyen I, Lefranc F, Bruyneel E, Dedecker R, Micik S, Brotchi J, Decaestecker C, Salmon I, Kiss R, Camby I: Molecular characterization of cell substratum attachments in human glial tumors relates to prognostic features. Glia 2001, 36:375–390
24. Chintala SK, Sawaya R, Gokaslan ZL, Fuller G, Rao JS: Immunohistochemical localization of extracellular matrix proteins in human glioma, both in vivo and in vitro. Cancer Lett 1996, 101:107–114
25. Hwang JH, Smith CA, Salhia B, Rutka JT: The role of fascin in the migration and invasiveness of malignant glioma cells. Neoplasia 2008, 10:149–159
26. Haugland HK, Tysnes BB, Tysnes OB: Adhesion and migration of human glioma cells are differently dependent on extracellular matrix molecules. Anticancer Res 1997, 17:1035–1042
27. Giese A, Rief MD, Loo MA, Berens ME: Determinants of human astrocytoma migration. Cancer Res 1994, 54:3897–3904
28. Joy AM, Beaudry CE, Tran NL, Ponce FA, Holz DR, Demuth T, Berens ME: Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis. J Cell Sci 2003, 116:4409–4417
29. Sasaki T, Giltay R, Talts U, Timpl R, Talts JF: Expression and distribution of laminin alpha1 and alpha2 chains in embryonic and adult mouse tissues: an immunochemical approach. Exp Cell Res 2002, 275:185–199
30. Rigot V, Lehmann M, André F, Daemi N, Marvaldi J, Luis J: Integrin ligation and PKC activation are required for migration of colon carcinoma cells. J Cell Sci 1998, 111:3119–3127
31. Osborne CK, Hobbs K, Clark GM: Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 1985, 45:584–590
32. Cruet-Hennequart S, Maubant S, Luis J, Gauduchon P, Staedel C, Dedhar S: av integrins regulate cell proliferation through integrin-linked kinase (ILK) in ovarian cancer cells. Oncogene 2003, 22:1688–1702
33. Chung J, Yoon S, Datta K, Bachelder RE, Mercurio AM: Hypoxia-induced vascular endothelial growth factor transcription and protection from apoptosis are dependent on alpha6beta1 integrin in breast carcinoma cells. Cancer Res 2004, 64:4711–4716
34. McComb RD, Bigner DD: Immunolocalization of laminin in neoplasms of the central and peripheral nervous systems. J Neuropathol Exp Neurol 1985, 44:242–253
35. Pedersen PH, Marienhagen K, Mork S, Bjerkvig R: Migratory pattern of fetal rat brain cells and human glioma cells in the adult rat brain. Cancer Res 1993, 53:5158–5165
36. Brem S, Cotran R, Folkman J: Tumor angiogenesis: a quantitative method for histologic grading. J Natl Cancer Inst 1972, 48:347–356
37. Chung J, Mercurio AM: Contributions of the alpha6 integrins to breast carcinoma survival and progression. Mol Cells 2004, 17:203–209
38. Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT: Angiogenesis in brain tumours. Nat Rev Neurosci 2007, 8:610–622
39. Leon SP, Folkerth RD, Black PM: Microvessel density is a prognostic indicator for patients with astroglial brain tumors. Cancer 1996, 77:362–372
40. Giese A, Bjerkvig R, Berens ME, Westphal M: Cost of migration:

invasion of malignant gliomas and implications for treatment. J Clin Oncol 2003, 21:1624–1636
41. Falcioni R, Kennel SJ, Giacomini P, Zupi G, Sacchi A: Expression of tumor antigen correlated with metastatic potential of Lewis lung carcinoma and B16 melanoma clones in mice. Cancer Res 1986, 46:5772–5778
42. Cress AE, Rabinovitz I, Zhu W, Nagle RB: The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. Cancer Metastasis Rev 1995, 14:219–228
43. Rabinovitz I, Nagle RB, Cress AE: Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. Clin Exp Metastasis 1995, 13:481–491
44. Raithatha SA, Muzik H, Rewcastle NB, Johnston RN, Edwards DR, Forsyth PA: Localization of gelatinase-A and gelatinase-B mRNA and protein in human gliomas. Neuro Oncol 2000, 2:145–150
45. Kachra Z, Beaulieu E, Delbecchi L, Mousseau N, Berthelet F, Moumdjian R, Del Maestro R, Beliveau R: Expression of matrix metalloproteinases and their inhibitors in human brain tumors. Clin Exp Metastasis 1999, 17:555–566
46. Freitas VM, Vilas-Boas VF, Pimenta DC, Loureiro V, Juliano MA, Carvalho MR, Pinheiro JJ, Camargo AC, Moriscot AS, Hoffman MP, Jaeger RG: SIKVAV, a laminin alpha1-derived peptide, interacts with integrins and increases protease activity of a human salivary gland adenoid cystic carcinoma cell line through the ERK 1/2 signaling pathway. Am J Pathol 2007, 171:124–138
47. Vajkoczy P, Goldbrunner R, Farhadi M, Vince G, Schilling L, Tonn JC, Schmiedek P, Menger MD: Glioma cell migration is associated with glioma-induced angiogenesis in vivo. Int J Dev Neurosci 1999, 17:557–563
48. Farin A, Suzuki SO, Weiker M, Goldman JE, Bruce JN, Canoll P: Transplanted glioma cells migrate and proliferate on host brain vasculature: a dynamic analysis. Glia 2006, 53:799–808
49. Debray C, Vereecken P, Belot N, Teillard P, Brion JP, Pandolfo M, Pochet R: Multifaceted role of galectin-3 on human glioblastoma cell motility. Biochem Biophys Res Commun 2004, 325:1393–1398